

# Premature aging and immune senescence in HIV-infected children

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**Objective:** Several pieces of evidence indicate that HIV-infected adults undergo premature aging. The effect of HIV and antiretroviral therapy (ART) exposure on the aging process of HIV-infected children may be more deleterious since their immune system coevolves from birth with HIV.

**Design:** Seventy-one HIV-infected (HIV+), 65 HIV-exposed-uninfected (HEU), and 56 HIV-unexposed-uninfected (HUU) children, all aged 0–5 years, were studied for biological aging and immune senescence.

**Methods:** Telomere length and T-cell receptor rearrangement excision circle levels were quantified in peripheral blood cells by real-time PCR. CD4<sup>+</sup> and CD8<sup>+</sup> cells were analysed for differentiation, senescence, and activation/exhaustion markers by flow cytometry.

**Results:** Telomere lengths were significantly shorter in HIV+ than in HEU and HUU children (overall,  $P < 0.001$  adjusted for age); HIV+ ART-naïve (42%) children had shorter telomere length compared with children on ART ( $P = 0.003$  adjusted for age). T-cell receptor rearrangement excision circle levels and CD8<sup>+</sup> recent thymic emigrant cells (CD45RA<sup>+</sup>CD31<sup>+</sup>) were significantly lower in the HIV+ than in control groups (overall,  $P = 0.025$  and  $P = 0.005$ , respectively). Percentages of senescent (CD28<sup>−</sup>CD57<sup>+</sup>), activated (CD38<sup>+</sup>HLA-DR<sup>+</sup>), and exhausted (PD1<sup>+</sup>) CD8<sup>+</sup> cells were significantly higher in HIV+ than in HEU and HUU children ( $P = 0.004$ ,  $P < 0.001$ , and  $P < 0.001$ , respectively). Within the CD4<sup>+</sup> cell subset, the percentage of senescent cells did not differ between HIV+ and controls, but programmed cell death receptor-1 expression was upregulated in the former.

**Conclusions:** HIV-infected children exhibit premature biological aging with accelerated immune senescence, which particularly affects the CD8<sup>+</sup> cell subset. HIV infection *per se* seems to influence the aging process, rather than exposure to ART for prophylaxis or treatment.

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## Introduction

The introduction of antiretroviral therapy (ART) has changed the natural history of pediatric HIV infection; ART-based prophylaxis regimens have in fact reduced mother-to-child transmission of HIV from 15–20% to under 2% in high-income countries, and have also given rise to substantial improvements in terms of survival and quality of life in HIV-infected children [1,2]. HIV infection is, therefore, now considered a chronic disease which persists for many decades [3]. However, despite improvements in immune function and reduction of AIDS-related complications, including opportunistic infections and AIDS-associated malignancies, ART does not restore full health. Many studies have demonstrated that ART-treated HIV-infected adults have a higher risk of non-AIDS-related overall morbidity and mortality compared with age-matched HIV-uninfected individuals. This increased risk is mainly because of a range of non-AIDS-defining illnesses associated with aging, including malignancies [4–8], and it has been advanced that the increase in non-AIDS-defining diseases among HIV-infected patients may be because of premature aging [9]. The pathogenic mechanism underlying this increased risk is still poorly understood. Chronic immune activation because of the persistence of circulating HIV virions may play a key role in the senescent pathway. Activated cells undergo clonal expansion in response to viral persistence, resulting in differentiation and accumulation of non-functional senescent cells [10]. It has been also advanced that premature and accelerated aging in HIV-infected patients can be because of adverse effects of antiretroviral drugs. Nucleoside reverse transcriptase inhibitors have been shown to inhibit telomerase activity in replicating cell lines *in vitro*, leading to accelerated shortening of telomere length [11,12].

The clinical complications of HIV infection and ART treatment in children may be more serious than in adults. The course of vertically transmitted infection in infants is characterized by faster disease progression and shorter time to AIDS, compared with adults. After infection, plasma HIV-RNA levels are higher in infants than in adults. In addition, they persist at high levels and decline slowly with age in the absence of ART [13,14], whereas in adults control of viral load is reached a few weeks after infection [15]. This slower control of viral replication is probably because of the fact that the immune system is still maturing. The innate immunity in children is of particular importance and plays a critical role in HIV pathogenesis [16–18], as the adaptive immune system is still developing [19]. HIV infection since birth together with long-term exposure to ART may affect premature aging and immune senescence in HIV-infected children even more than in adults.

To date, few data are available on premature aging in HIV-infected children [20–23] and no reports give a

comprehensive assessment of telomere shortening, a key molecular marker of biological aging, together with the activation/senescent profile of T lymphocytes. In this study, we analysed biological aging in relation to immune activation and senescence markers in a cohort of perinatally HIV-infected children.

## Methods

### Ethic statement

The study was approved by the Ethics Committees of the Azienda Ospedaliera Padova (Prot. n.#2921P) and the Hospital Sant Joan de Déu, Universitat de Barcelona (Prot. n.#04–15); written informed consent was obtained for all children from their parents/guardians.

### Study population

A total of 71 perinatally HIV-infected (HIV+), 65 HIV-exposed-uninfected (HEU), and 56 HIV-unexposed-uninfected (HUU) children, all aged 0–5 years, were included in this study. HIV+ and HEU children attended the Department of Mother and Child Health, University of Padova or the Infectious Diseases Unit, Pediatrics Department, Hospital Sant Joan de Déu, Universitat de Barcelona. For each HIV+ and HEU child, the first cryopreserved sample available after birth was chosen for the study. None of the HIV+ or HEU children was breastfed. HUU children were recruited at Pediatric Emergency Department of Azienda Ospedaliera Padova or Hospital Sant Joan de Déu, Universitat de Barcelona. Exclusion criteria were malignancies, chronic infections, sarcoidosis, diabetes mellitus type-1, rheumatoid arthritis, and systemic lupus erythematosus.

### Sample preparation

Peripheral blood mononuclear cells (PBMC) were isolated from ethylenediaminetetraacetic acid-treated peripheral blood (2–5 ml) by centrifugation on a Ficoll-Paque gradient (Pharmacia, Uppsala, Sweden). PBMC were cryopreserved and plasma samples were stored in liquid nitrogen and at –80°C, until use.

### Telomere length measurement by quantitative real-time PCR

Relative telomere length was determined by monochrome quantitative multiplex PCR assay [24] with minor modifications. Each PCR reaction was performed in a final volume of 25 µl containing 5 µl sample (2 ng DNA/µl) and 20 µl reaction mix containing 0.75 × SYBR GreenI (Invitrogen, Italy), 10 mmol/l Tris-hydrochloric acid pH 8.3, 50 mmol/l potassium chloride, 3 mmol/l magnesium chloride, 0.2 mmol/l each deoxynucleotide (dNTP) (Applied Biosystems, Foster City, California, USA), 1 mmol/l dithiothreitol, 0.625 U AmpliTaq Gold DNA polymerase, 1% dimethyl sulfoxide (Sigma-Aldrich, St Louis, Missouri, USA), and

900 nmol/l of each of the primers. Telomere and albumin gene primers sequences are described in [24]. PCR reactions were performed on a LightCycler480 real-time PCR detection system (Roche Applied Science, Mannheim, Germany). The thermal cycling profile was 15 min at 95°C, two cycles of 15 s at 94°C, 15 s at 49°C, followed by 40 cycles of 15 s at 94°C, 10 s at 62°C, 15 s at 74°C, 10 s at 84°C, 15 s at 88°C, with signal acquisition at the end of both the 74°C and 88°C steps. A standard curve was generated at each PCR run, consisting of DNA from the RAJI cell line, serially diluted from 100 to 0.41 ng/μl [25]. LightCycler raw text files were converted using the LC480Conversion free software (<http://www.hartfaalcentrum.nl/index.php?main=files&fileName=LC480Conversion.zip&description=LC480%20Conversion&sub=LC480Conversion>), and the data were analysed using LinRegPCR free software [26]. All samples were blindly and consecutively run in triplicate together with reference samples. The intra and interassay reproducibility of both telomere and albumin PCR results was evaluated using dilutions of the reference curve. Telomere length values were calculated as telomere/single-copy gene (T/S) ratio, as previously described [25]. The intra and interassay variability of T/S values was evaluated using reference samples; coefficients of variation were 3.98% or less and 8.14% or less, respectively.

### T-cell receptor rearrangement excision circle levels quantification

Thymic output in PBMC was studied by measurement of T-cell receptor rearrangement excision circle (TREC) levels by real-time PCR, as previously described [27]. TREC levels were expressed as the number of TREC copies/10<sup>5</sup> PBMC.

### Viral load quantification

Plasma HIV-RNA levels were determined in all HIV-infected children using the COBAS Taqman HIV-1 test (Roche, Branchburg, New Jersey, USA). The lower limit of detection was 50 HIV-RNA copies/ml. HIV-DNA levels in PBMC were measured by real-time PCR, and expressed as HIV-DNA copies/10<sup>6</sup> PBMC as previously described [27].

### Flow cytometry analysis

T-cell phenotyping was performed on cryopreserved PBMC. Cells were thawed, washed, stained for 20 min in the dark with the Live/Dead Fixable Near-IR Dead Cell Stain Kit (Life Technologies, Carlsbad, California, USA) and with fluorescent-conjugated mononuclear antibodies CD3-fluorescein isothiocyanate, CD31-phycoerythrin (PE), CD38-PE, CD57-PE, CD45RA-allophycocyanin (APC) and programmed cell death receptor (PD)-1-PECy7 (Becton-Dickinson, San Diego, California, USA), CD27-PECy7 (Beckman Coulter, Fullerton, California, USA) and CD4-VioBlue, CD8-VioGreen, Human Leukocyte Antigen - antigen

D Related (HLA-DR)-APC, and CD28-APC (Miltenyi Biotec, Auburn, California, USA). Appropriate isotypic controls (mouse IgG1-PE, IgG2b-APC, and IgG1k-PECy7) were used to evaluate nonspecific staining. Cells were then washed and resuspended in phosphate-buffered saline supplemented with 1% paraformaldehyde. All samples were analysed using LSR II Flow cytometer (Becton-Dickinson). A total of 100 000 events were collected in the lymphocyte gate using morphological parameters (forward and side-scatter). Data were processed with FACSDiva Software (Becton-Dickinson) and analysed using Kaluza Analyzing Software v.1.2 (Beckman Coulter) (Supplementary Figure 1, <http://links.lww.com/QAD/A903>). Samples for flow-cytometry analysis were available for 24 HIV+ children, 21 HEU, and 18 HUU children. The characteristics of these subgroups are given in Supplementary Table 1, <http://links.lww.com/QAD/A904>.

### Quantification of soluble markers

Plasma levels of soluble CD14 (sCD14), IL-6, and TNFα were quantified with commercially available assays (Human sCD14, IL-6, and TNFα Quantikine ELISA; R&D Systems, Minneapolis, Minnesota, USA) according to the manufacturer's protocol. Samples for analysis of soluble markers were available for 24 HIV+, 21 HEU, and 18 HUU children.

### Statistical analysis

Unadjusted comparisons of continuous variable distributions among groups were assessed with the Kruskal-Wallis nonparametric test, and the associations between categorical variables were analysed by the  $\chi^2$  test. Spearman's  $\rho$  coefficient ( $r_s$ ) was used for correlations. Normal distributions for telomere length and TREC levels were visually checked by quantile-quantile plots. Linear regression models estimated the telomere length and TREC levels in HIV+, HEU, and HUU children, ART exposure and naive groups, log-transformed HIV-RNA and HIV-DNA covariates, adjusted for age and its interaction with groups. Samples with undetectable plasma HIV-RNA were assigned a value of 20 copies/ml to include them in the statistical analyses. All statistical analyses were performed with Statistical Analysis Software (SAS) (Release 9.2; SAS Institute, Cary, North Carolina, USA). Adjustments for multiple testing with Hochberg's correction were made for comparisons of CD4<sup>+</sup> and CD8<sup>+</sup> cell subsets among groups. All *P* values were two-tailed, and were considered significant at less than 0.05.

## Results

### Characteristics of study population

The characteristics of the study population are listed in Table 1. The median age of HIV+ children was 3.11

[interquartile range (IQR) 1.41–4.48], 1.73 (0.99–3.20) for HEU, and 1.85 (0.85–3.46) years for HUU children. Thirty of the 71 (42%) HIV+ children were ART-naïve, the others were on ART [median time 18 (11–36.5) months]. ART-naïve HIV+ children were younger than those on therapy [1.70 (0.69–3.59) and 3.62 (2.17–4.81) years, respectively;  $P=0.004$ ]. Sixty-one of the 65 (93.8%) HEU children had been exposed to ART prophylaxis during gestation [median 34 (17–38) weeks] and received postnatal prophylaxis [6 weeks of zidovudine (ZDV) monotherapy]. Of the 61 ART-treated pregnant women, 40 (65.6%) had received triple therapy based on ZDV + lamivudine (3TC) + nevirapine (NVP), 17 (27.8%) ZDV + 3TC + protease inhibitor, and the remaining 4 (6.6%) a regimen based on tenofovir (TDF) + emtricitabine (FTC) + protease inhibitor.

### Telomere length is shorter in HIV-infected children than in controls

The median telomere length value in PBMC was significantly lower in HIV+ than in HEU and HUU children, being 2.21 (1.94–2.58), 2.63 (2.25–3.21), and 2.88 (2.49–3.1), respectively [ $P<0.001$  not age adjusted (Fig. 1a);  $P<0.001$  age adjusted]. Telomere length values significantly decreased with age in HEU and HUU (regression coefficient ( $\beta$ ) =  $-0.0102$ ,  $P=0.008$  and  $\beta = -0.0100$ ,  $P=0.011$ , respectively), but not in HIV+ children ( $\beta = -0.0018$ ,  $P=0.587$ ) (Fig. 1b). Of note, in the HIV+ group, ART-naïve children had shorter telomere length compared with those on ART [2.11 (1.75–2.37) ‘vs.’ 2.46 (2.07–2.68);  $P=0.006$  not age adjusted (Fig. 1c);  $P=0.003$  age adjusted]. Telomere lengths were not associated with age in either ART-naïve ( $\beta = -0.0054$ ,  $P=0.227$ ) or ART-treated children ( $\beta = -0.0046$ ,  $P=0.258$ ) (Fig. 1d). After adjusting for age, mean telomere length values tended to decrease with increasing HIV-RNA levels ( $\beta = -0.0396$ ;  $P=0.056$ ),

but not with HIV-DNA levels ( $\beta = -0.0004$ ,  $P=0.490$ ), and were significantly lower in ART-naïve children ( $\beta = -0.3913$ ,  $P=0.003$ ). In the final multivariate model, including all variables, the stepwise method selected only ART exposure as a significant predictor variable of higher mean telomere length value ( $P=0.039$ ).

### Thymic output is lower in HIV-infected children than in controls

HEU and HUU children had higher TREC levels than HIV+ children [5409 (3470–6600), 5370 (2380–8102), 3498 (2051–6780)] TREC copies/ $10^5$  PBMC, respectively [ $P=0.018$  not age adjusted (Fig. 1e);  $P=0.025$  age-adjusted]. TREC levels decreased significantly with increasing age in HEU and HUU groups ( $\beta = -61$ ,  $P=0.009$  and  $\beta = -86$ ,  $P=0.001$ , respectively), but not in HIV+ children ( $\beta = -17$ ,  $P=0.353$ ) (Fig. 1f). No significant differences in TREC dynamics were found between ART-treated and ART-naïve children (Fig. 1g and h).

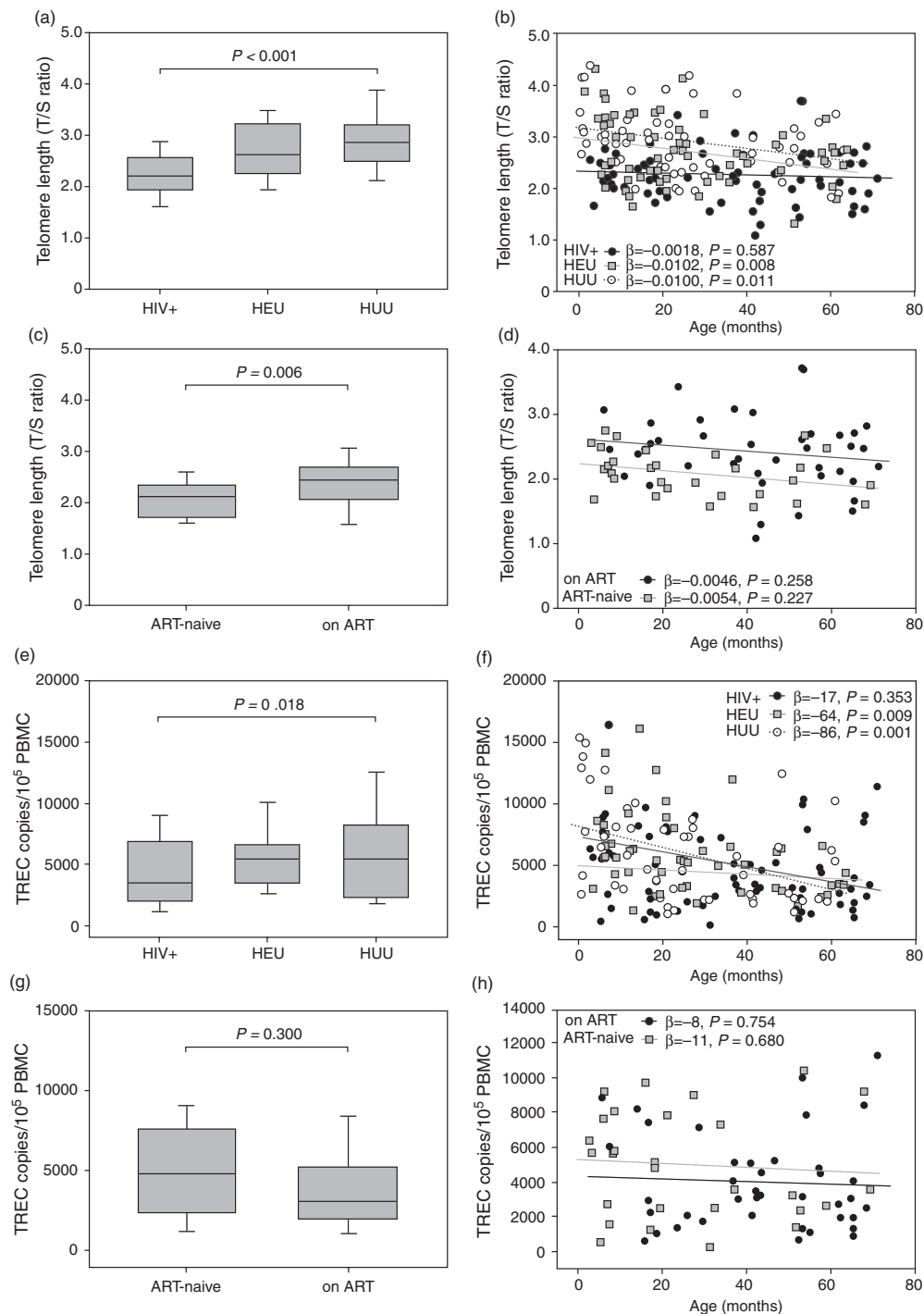
### Phenotypic T-cell alterations occur early in HIV-infected children

There were no differences in the frequencies of CD3<sup>+</sup> cells among the three groups ( $P=0.590$ ) (Table 2). The percentages of total CD4<sup>+</sup> cells were lower in the HIV+ than in the control groups ( $P=0.001$ ). Within CD4<sup>+</sup> cells, HIV+ and control groups did not significantly differ in percentages of naïve (CD45RA<sup>+</sup>CD27<sup>+</sup>), central memory (CD45RA<sup>+</sup>CD27<sup>+</sup>), effector memory (CD45RA<sup>+</sup>CD27<sup>+</sup>), and terminally differentiated (CD45RA<sup>+</sup>CD27<sup>+</sup>) cell subsets (Table 2). However, when central and effector memory cell subsets, the major cellular reservoirs for HIV [28], were considered together, they tended to be lower in HIV+ than in HEU and HUU children [21.9 (15.3–38.3)% ‘vs.’ 29.8

**Table 1. Demographic and clinical characteristics of HIV+, HIV-exposed-uninfected children, and HIV-unexposed-uninfected children.**

	HIV+ ( $n=71$ )	HEU ( $n=65$ )	HUU ( $n=56$ )
Age, median (IQR) years	3.11 (1.40–4.48)	1.74 (0.99–3.31)	1.85 (0.84–3.46)
Sex, $n$ (%)			
Men	39 (55%)	34 (52%)	29 (52%)
Ethnicity/race, $n$ (%)			
White	49 (69%)	47 (72.3%)	49 (87.5%)
Black	20 (28.2%)	15 (23.1%)	5 (9.0%)
Asian	2 (2.8%)	3 (4.6%)	2 (3.5%)
Exposed to ART prophylaxis, $n$ (%)	5 (7%)	61 (93.8%)	–
Exposed to ART, $n$ (%)	41 (58%)	–	–
Duration of ART exposure, median (IQR) months	18 (12–36)	–	–
Percentage of lifetime on ART	57.5 (42.6–84.5)	–	–
Detectable plasmaviremia at sample collection, $n$ (%)	54/71 (76.1%)	–	–
ART naïve	30/30 (100%)	–	–
On ART	17/41 (58.5%)	–	–
Plasmaviremia at sample collection ( $\log_{10}$ copies/ml)			
ART naïve	5.31 (4.90–5.62)	–	–
On ART	3.96 (2.70–5.27)	–	–

ART, antiretroviral therapy; HEU, HIV-exposed-uninfected children; HIV+, HIV-infected children; HUU, HIV-unexposed-uninfected children; IQR, interquartile range.



**Fig. 1. HIV-infected (HIV+) children have shorter telomere length (TL) and lower T-cell receptor rearrangement excision circle (TREC) levels than HIV-exposed-uninfected (HEU) and HIV-unexposed-uninfected (HUU) children.** (a) TL values not age-adjusted in HIV+ ( $n = 71$ ), HEU ( $n = 65$ ), and HUU ( $n = 56$ ) children. (b) TL as function of age in HIV+ (black circles, continuous line), in HEU (gray squares, continuous line), and HUU (white circles, dotted line) children. (c) TL values not age-adjusted in HIV+ children, subdivided into antiretroviral therapy (ART)-naive ( $n = 30$ ) and on ART ( $n = 41$ ). (d) TL as function of age in HIV+ children, subdivided into ART-naive (gray squares) and ART-treated (black circles). (e) TREC levels not age-adjusted in HIV+ ( $n = 71$ ), HEU ( $n = 65$ ), and HUU ( $n = 56$ ) children. (f) TREC levels as function of age in HIV+ (black circles, continuous line), in HEU (gray squares, continuous line) and HUU (white circles, dotted line) children. (g) TREC levels not age-adjusted among HIV+ children, subdivided into ART-naive ( $n = 30$ ) and on ART ( $n = 41$ ). (h) TREC levels as function of age in HIV+ children, subdivided into ART-naive (gray squares) and on ART (black circles). Boxes and whiskers: 25–75th and 10–90th percentiles, respectively; central line in boxes: median.  $\beta$ , regression coefficient.

**Table 2. Frequencies of CD4<sup>+</sup> and CD8<sup>+</sup> T-cell subsets.**

	HIV+ ( <i>n</i> = 24) % median (IQR)	HEU ( <i>n</i> = 21) % median (IQR)	HUU ( <i>n</i> = 18) % median (IQR)	Overall <i>P</i> value
CD3 <sup>+</sup>	64.4 (58.8–69.2)	61.8 (49.8–68.4)	60.2 (54.6–66.4)	0.590
CD4 <sup>+</sup>	39.6 (35.1–45.3)	53.4 (43.5–64.7)	52.5 (38.0–60.6)	0.001
Naive	76.8 (59.9–84.4)	70.1 (59.9–81.0)	64.7 (56.9–75.3)	0.423
Central memory	18.1 (13.2–31.1)	27.3 (17.4–32.6)	27.9 (20.7–35.1)	0.206
Effector memory	3.4 (1.2–5.8)	2.5 (1.8–6.3)	4.4 (2.9–7.8)	0.220
T. differentiated	0.5 (0.2–1.0)	0.3 (0.1–0.6)	0.4 (0.3–0.1)	0.330
RTE	63.6 (54.9–72.4)	58.3 (46.0–68.2)	54.2 (49.3–61.7)	0.181
PEC	12.4 (5.3–16.4)	11.7 (8.2–16.1)	10.4 (8.1–15.2)	0.738
Senescent	0.4 (0.1–0.7)	0.2 (0.1–0.5)	0.2 (0.1–1.3)	0.568
Activated	3.3 (2.2–5.9)	2.1 (1.3–3.5)	2.8 (1.6–3.8)	0.041
Exhausted	4.1 (3.4–6.6)	3.5 (1.9–5.2)	3.2 (2.7–5.1)	0.050
CD8 <sup>+</sup>	31.2 (25.8–39.6)	28.9 (21.2–35.0)	25.9 (22.8–29.0)	0.063
Naive	46.2 (37.6–75.1)	77.1 (55.9–84.7)	71.0 (46.7–86.1)	0.019
Central memory	11.0 (6.9–23.2)	16.3 (10.9–26.5)	12.7 (9.8–18.9)	0.278
Effector memory	7.1 (2.3–13.1)	2.1 (1.1–4.9)	4.3 (1.3–11.4)	0.033
T. differentiated	16.3 (4.5–36.4)	2.5 (1.0–8.6)	4.2 (2.1–16.2)	0.001
RTE	55.3 (41.4–71.8)	69.8 (60.4–80.1)	68.1 (59.5–79.3)	0.005
PEC	17.1 (6.5–29.2)	5.9 (3.8–15.3)	9.8 (4.6–15.2)	0.040
Senescent	25.8 (12.4–43.2)	8.5 (6.8–16.7)	9.7 (3.3–27.3)	0.004
Activated	7.0 (5.2–12.2)	4.7 (3.9–7.6)	3.4 (2.8–6.7)	<0.001
Exhausted	7.1 (5.0–12.4)	3.5 (2.1–5.9)	3.7 (2.4–5.3)	<0.001

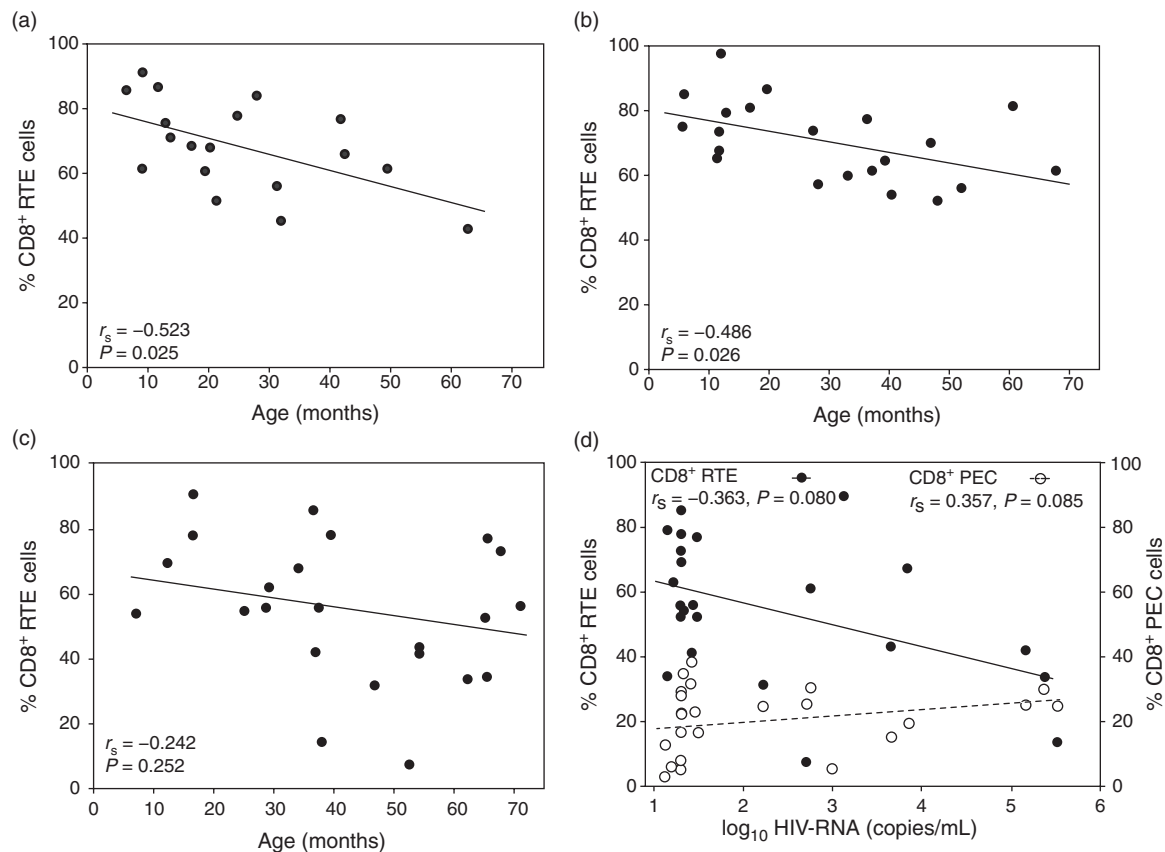
HEU, HIV-exposed-uninfected children; HIV+, HIV-infected children; HUU, HIV-unexposed-uninfected children; IQR, interquartile range; PEC, peripheral expanded cells; RTE, recent thymic emigrant cells.

(18.6–39.4)% and 35.6 (26.2–42.6)%;  $P=0.085$ ]. The percentages of senescent cells (CD4<sup>+</sup>CD28<sup>−</sup>CD57<sup>+</sup>) were similar in the three groups, whereas activated CD38<sup>+</sup>HLA-DR<sup>+</sup> and exhausted PD-1<sup>+</sup> were more expanded in HIV+ children than in controls (Table 2). In particular, within the HIV+ group, the percentages of CD4<sup>+</sup>CD38<sup>+</sup>HLA-DR<sup>+</sup> and CD4<sup>+</sup>PD-1<sup>+</sup> were higher in children with detectable viral load than in aviremic children ( $P=0.056$  and  $P=0.037$ , respectively).

The median of CD8<sup>+</sup> cell percentages tended to be higher in HIV+ children than in control groups ( $P=0.063$ ). Notably, significant differences emerged among CD8<sup>+</sup> T-cell subsets. HIV+ children showed lower percentage of naive cells than HEU and HUU children [46.2 (37.6–75.1)%, 77.1 (55.9–84.7)%, 71.0 (46.7–86.1)%;  $P=0.019$ ]. In particular, HIV+ children had a lower frequency of CD8<sup>+</sup> recent thymic emigrant [recent thymic emigrant cells (RTE), CD45RA<sup>+</sup>CD31<sup>+</sup>] cells ( $P=0.005$ ) and a higher percentage of peripheral expanded cells [peripheral expanded cells (PEC) CD45RA<sup>+</sup>CD31<sup>−</sup>] than control groups ( $P=0.040$ ), suggesting strong peripheral cell proliferation (Table 2). In addition, the percentage of CD8<sup>+</sup> RTE cells decreased with age in HEU and HUU but not in HIV+ children (Fig. 2a–c). Interestingly, CD8<sup>+</sup> RTE cells were lower in children with detectable viral load than in aviremic children [41.8 (22.6–64.4)% ‘vs.’ 55.9 (53.6–76.7)%;  $P=0.039$ ] (not shown); plasma HIV-RNA tended to be inversely correlated with CD8<sup>+</sup> RTE cells ( $r_s=-0.363$ ,  $P=0.080$ ) and positively correlated with CD8<sup>+</sup> PEC ( $r_s=0.357$ ,  $P=0.085$ ) (Fig. 2d).

Among memory cell subsets, the frequency of CD8<sup>+</sup> central memory did not differ among HIV+, HEU, and

HUU children ( $P=0.278$ ). However, in the HIV+ group, this subset was more expanded in children with detectable viremia than in those with undetectable HIV-RNA ( $P=0.027$ ) (not shown). Both effector memory (CD45RA<sup>−</sup>CD27<sup>−</sup>) and terminally differentiated cells (CD45RA<sup>+</sup>CD27<sup>−</sup>) were more expanded in HIV+ children than in control groups (Table 2). In HIV+ children, the proportion of senescent CD8<sup>+</sup> cells was also higher than in HEU and HUU groups [25.8 (12.4–43.2)% ‘vs.’ 8.5 (6.8–16.7)% and 9.7 (3.3–27.3)%;  $P=0.004$ ]. This expansion was particularly observed in children with detectable plasmaviremia, indicating that active HIV replication stimulates the production of a senescent phenotype [41.6 (18.5–45.8)% ‘vs.’ 4.4 (2.1–13.4)%;  $P=0.002$ ]. In addition, the activation of CD8<sup>+</sup> cells was significantly higher in HIV+ children than in controls [7.0 (5.2–12.2)% ‘vs.’ 4.7 (3.9–7.6)% in HEU and 3.4 (2.8–6.7)% in HUU;  $P<0.001$ ] (Table 2), and significantly increased with HIV-RNA levels ( $r_s=0.672$ ;  $P<0.001$ ) (not shown). The percentages of CD8<sup>+</sup>PD-1<sup>+</sup> cells were significantly higher in HIV+ than in HEU and HUU children ( $P=0.003$  and  $P<0.001$ , respectively) (Table 2). After adjustments for multiple testing, the CD4<sup>+</sup> cell subset, and the CD8<sup>+</sup> terminally differentiated, activated, exhausted, senescent, and RTE cell subsets remained significantly different ( $P<0.05$ ). Also, the levels of proinflammatory cytokines IL-6 and TNFα associated with the senescent phenotype [29–31] were higher in HIV+ than in HEU and HUU children (Supplementary Table 2, <http://links.lww.com/QAD/A905>), thus supporting the accelerated immune senescence in HIV+ children. PD-1 expression in viremic subjects was also significantly higher than in those with undetectable plasmaviremia ( $P=0.002$ ) and was correlated with HIV-RNA levels ( $r_s=0.471$ ,  $P=0.021$ ) and



**Fig. 2. Relationship between recent thymic emigrant (RTE) CD8<sup>+</sup> cells with age and HIV plasmaviremia.** Percentage of CD8<sup>+</sup> RTE cells as function of age in (a) HUU, (b) HEU, and (c) HIV+ children. (d) Levels of HIV plasmaviremia in relation to percentages of CD8<sup>+</sup> RTE (black circles, continuous line) and CD8<sup>+</sup> peripheral expanded cells (PEC) (white circles, dotted line) among HIV+ children.  $r_s$ , Spearman's  $\rho$  correlation coefficient.

CD8<sup>+</sup>CD38<sup>+</sup>HLA-DR<sup>+</sup> cells ( $r_s = 0.528$ ,  $P = 0.009$ ) (not shown).

Telomere length values were inversely correlated with percentages of senescent (Fig. 3a), activated (Fig. 3d) and exhausted CD8<sup>+</sup> cells (Fig. 3g) in HIV+, but not in HEU (Fig. 3b, e, h) or HUU children (Fig. 3c, f, i).

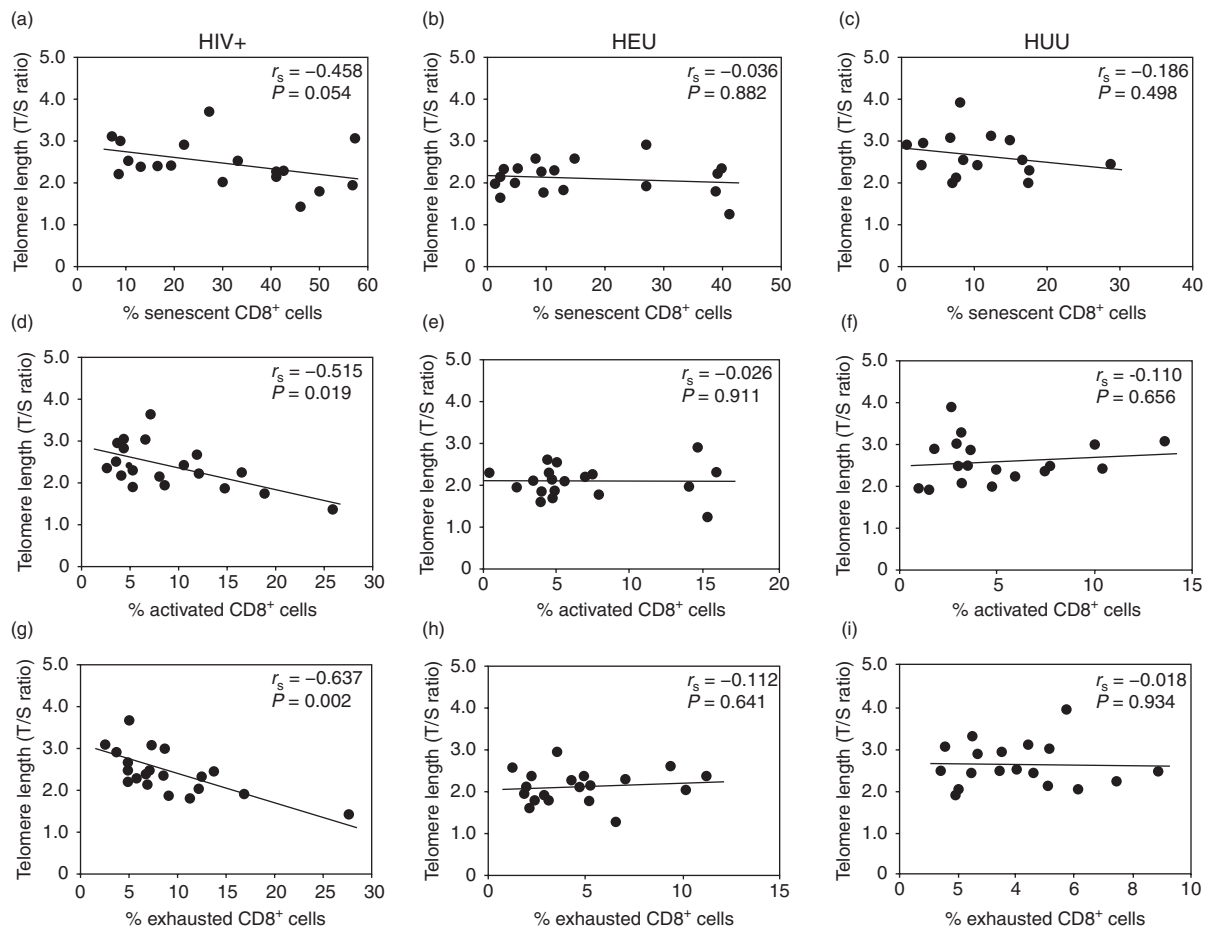
### HIV-infected children have increased gut microbial translocation

Median levels of sCD14 were significantly higher in HIV+ than in HEU and HUU children [2699 (2333–2826) ‘vs.’ 2138 (1954–2427) and 2065 (1850–2480) ng/ml;  $P < 0.001$ ] (Supplementary Figure 2A, <http://links.lww.com/QAD/A903>). In HIV+ children, sCD14 levels positively correlated with HIV-RNA levels ( $r_s = 0.585$ ,  $P = 0.007$ ), with percentages of CD8<sup>+</sup>HLA-DR<sup>+</sup>CD38<sup>+</sup> ( $r_s = 0.418$ ,  $P = 0.065$ ), and CD8<sup>+</sup>PD-1<sup>+</sup> ( $r_s = 0.645$ ,  $P = 0.002$ ), but not with percentages of CD8<sup>+</sup>CD28<sup>−</sup>CD57<sup>+</sup> ( $r_s = 0.172$ ,  $P = 0.487$ ) or telomere length ( $r_s = -0.386$ ,  $P = 0.124$ ) (Supplementary Figure 2b–f, <http://links.lww.com/QAD/A903>).

## Discussion

This is the first study describing biological aging and immune senescence in HIV-infected children compared with HIV-exposed-uninfected and unexposed-uninfected children, all aged 0–5 years. Overall, the results demonstrate that HIV-infected children exhibit premature biological aging with accelerated immune senescence which affects the CD8<sup>+</sup> T-cell subset in particular.

In contrast to the study of Côté *et al.* [21], which found no difference in telomere length between HIV+ children and controls, in our study telomere length was significantly shorter in HIV+ than in HEU and HUU children. This discordant result may be because of the different ages of the two cohorts: the children enrolled in our study were younger (aged 0–5 years, median 3.1) than those of Côté *et al.* (aged 0–19 years, median 13.3). As telomere shortening in peripheral blood cells is very rapid during the first years of life [32], the difference between HIV-infected children and controls may have emerged more clearly in our cohort. The two cohorts also differed in duration of ART exposure. The longer



**Fig. 3. Relationship between telomere length (TL) and T-cell immunophenotypic profiles.** TL values in relation to percentages of senescent (CD8<sup>+</sup>CD28<sup>-</sup>CD57<sup>+</sup>) cells in (a) HIV+, (b) HEU, and (c) HUU children, activated (CD8<sup>+</sup>CD38<sup>+</sup>HLA-DR<sup>+</sup>) cells in (d) HIV+, (e) HEU, and (f) HUU children, and exhausted (CD8<sup>+</sup>PD-1<sup>+</sup>) cells in (g) HIV+, (h) HEU, and (i) HUU children. HIV+, HIV-infected; HEU, HIV-exposed uninfected; HUU, HIV-unexposed uninfected;  $r_s$ , Spearman's  $\rho$  correlation coefficient.

exposure to ART of children in the above study (median 85 months of ART exposure) may explain the loss of association found in our study population, consisting of ART-naïve children or ones recently on ART (median 18 months of ART exposure).

Nucleoside reverse transcriptase inhibitors are known inhibitors of telomerase reverse transcriptase, and have been reported to be associated with telomere shortening [11,12]. The inverse association between telomere length and ART-exposure (median time 123 months) has recently been demonstrated in a cohort of adults, but the small sample size and lack of optimal control of ART-naïve patients preclude definite conclusions [33]. Two other studies of HIV-infected adults [34,35] confirm the association of telomere length shortening with HIV status, but not with relatively short ART exposure (median times 58 and 48 months, respectively).

In our group of HIV-infected children, the ART-naïve ones, even though younger than those on ART, had

significantly shorter telomeres than the latter. All these findings indicate that HIV infection *per se*, rather than exposure to therapy, influences the aging process. Also, the finding that over 90% of HEU children had been perinatally exposed to ART and that their telomere length did not differ from those of HUU children indicates that relatively short-term ART exposure does not significantly influence telomere length, matching the results of a recent report [36]. It is possible that longer exposure to ART in HIV-infected children exacerbates HIV-driven telomere shortening.

The mechanism behind telomere shortening in HIV-infected individuals is unknown. Short telomeres may be because of excessive cellular replication after chronic immune activation, but the virus itself may play an active role. Telomerase activity is in fact severely impaired in uninfected CD34<sup>+</sup> hematopoietic progenitor cells isolated from HIV-infected patients [37]. HIV infection and HIV-Tat protein also downmodulate telomerase expression and activity in lymphoblastoid cells [38] and



peripheral blood lymphocytes [39–41]. Although the CD4<sup>+</sup> T cell is the target of HIV infection, we found that CD8<sup>+</sup> T-cell compartment was largely impaired in the HIV-infected children. They had a lower frequency of CD8<sup>+</sup> naive cells than controls and a decline in this cell subset did not correlate with age, as occurs in HIV-uninfected children. In particular, the decreased percentages of RTE cells with increased percentages of PEC together with increasing levels of HIV plasmaviremia indicate that HIV induces peripheral proliferation of CD8<sup>+</sup> cells and their differentiation into effector cells, which play a central role in immunity against pathogens [42]. As already described in adults and older children [22,43,44], the decrease in naive cell subset is associated with skewed maturation of CD8<sup>+</sup> cells toward an effector phenotype which, without adequate replenishment of new CD8<sup>+</sup> naive cells, induces accumulation of cells with a senescent phenotype. A major driver of the cellular senescent phenotype is telomere shortening [29]. Our data indicate that HIV-infected children accumulate CD8<sup>+</sup>CD38<sup>+</sup> and CD8<sup>+</sup>PD-1<sup>+</sup> cells together with a higher percentage of senescent CD8<sup>+</sup> cells. The finding that activated and exhausted CD8<sup>+</sup> cells are inversely correlated with telomere length supports the idea that persistent immune activation and cellular exhaustion are closely linked to accelerated biological aging and immune senescence. Chronic viral coinfections may induce immune activation and accelerate immune senescence [35,45,46]. In particular, it has been shown that cytomegalovirus (CMV) leads to significant changes in the CD8<sup>+</sup> repertoire [45,46]. Unfortunately, clinical data on CMV serology were available only for HIV+ and a subgroup of HEU children: 40 of 71 (56.3%) HIV+ and nine of 33 (27.3%) HEU children were CMV-positive. The CMV+ and CMV- subgroups of HIV-infected children did not significantly differ as regards telomere length and markers of immunological profile. Larger studies are needed to better understand the contribution of coinfections in the aging process of HIV-infected children.

The finding that sCD14 levels were correlated with percentages of activated/exhausted CD8<sup>+</sup> cells and the lack of correlation between sCD14 and senescent CD8<sup>+</sup> cells and/or telomere length suggest that the gut damage and gastrointestinal lymphocyte depletion which occurs during HIV infection do not directly cause immune senescence, but they do contribute to accelerated aging and senescence through immune system activation. Overall, our data support a scenario in which viremia leads to high turnover with continual loss and output of naïve cells, which rapidly differentiate and exhaust their effector function, resulting in an accumulation of senescent cells with short telomeres. These data are consistent with a previous report [47] showing that high percentage of CD8<sup>+</sup>CD28<sup>-</sup> correlates with shorter telomeres.

The evidence that immune senescence is more serious in children with detectable HIV viremia focuses attention on the need for early and long-standing control of HIV replication and chronic immune activation. In particular, this aspect must be considered when a treatment interruption has been planned [48]. As previously demonstrated in a pediatric cohort, viremia does impair the immune reconstitution of memory and effector CD4<sup>+</sup> T-cell subsets [49], and also contributes to the expansion of T-regulatory cells [50] which may influence the specific HIV immune response, allowing the virus to replicate and increase immune activation.

In conclusion, HIV-infected children exhibit premature biological aging with accelerated immune senescence, which particularly affects the CD8<sup>+</sup> T-cell subset. The shorter telomere length and higher percentage of senescent cells in HIV+ children, compared with children on ART or HEU, suggest that HIV infection *per se*, rather than exposure to ART for prophylaxis or treatment, influences the aging process. These data support the importance of maintaining undetectable viral load to avoid premature immune senescence and dysfunction of CD8<sup>+</sup> cells, compromising their tumor immune surveillance function and increasing the risk of age-related diseases, including malignancies.

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Authors' contributions: K.G. designed and performed the experiments, undertook clinical data collection, data analysis and interpretation, wrote the first draft, carried out critical revision and provided intellectual input to further drafts. A.N.J. and C.F. provided clinical samples and clinical data, carried out critical revision and provided intellectual input to further drafts. O.R., E.M., and M.C. provided clinical samples and clinical data. P.D.B. provided statistical expertise and data analysis. Z.M., M.R.P., and R.F. performed the experiments. C.G. carried out critical revision and provided intellectual input to further drafts. A.D.R. conceived and designed the study, carried out data analysis and interpretation, undertook critical revision and provided intellectual input to further drafts. All the authors approved the final version of this article.

## Conflicts of interest

There are no conflicts of interest.

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